Recent Advancements in Nitrogen Use Efficiency in Crop Plants Achieved by Genomics and Targeted Genetic Engineering Approaches

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Abstract

Nitrogen plays a crucial role in plant metabolism, growth, and development of plants, and its deficiency leads to severe growth retardation and reduced grain yield. The efficient utilization of nitrogenous fertilizers is needed to enhance crop yield and also to fetch the food demand of the world population. The accumulated nitrogen in the ecosystem leads to severe environmental pollution and health hazards to inhabited animals. However, nitrogen inside plants is regulated by a set of nitrogen metabolism genes, promoters, and transcription factors. Further, the identification and characterization of nitrogen metabolism genes in crop plants is a prerequisite for developing tailored crop plants for increased nitrogen use efficiency (NUE), grain yield, biomass, and other economic traits. Moreover, NUE is a complex trait, and breeding crops for improving NUE is still in the infancy stage. Therefore, a targeted and holistic approach is required for enhanced nitrogen uptake and its utilization. The precise modulation of key genes of nitrogen metabolism, amino acid biosynthesis, and carbon metabolism could result in enhancement of NUE, and the engineered crop plants for NUE traits were reported to be superior in terms of NUE and also incurred higher grain yield, biomass, and improved agronomical parameters as that of cultivated crop cultivars. In this review, we described the basics of nitrogen metabolism, genomics, and recently targeted genetic engineering strategies employed in crop plants for improving NUE.

Keywords Nitrogen fertilizers · NUE · Genetic engineering · Genetically modified crop plants

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Introduction

The last four decades witnessed a double enhancement in cereal production driven mainly by the use of high-yielding varieties and fertilizers in agriculture (Zhu et al. 2022). Nitrogen (N) shares 78% of the atmospheric gaseous composition and crops are unable to use it in this molecular form (Anas et al. 2020). Although leguminous crops fix the gaseous N into a utilizable form such as ammonia (NH_3) and nitrate (NO_3^-), this fixed N is insufficient to meet the crop production demand of larger acreages (Ladha et al. 2022). Moreover, N is deficient in almost all agricultural soils of the world, and about 111.59 million tonnes of nitrogenous fertilizers are applied in world agricultural land to meet the crop's N requirement (FAO 2022). To tackle N paucity, the farmers generally solicit N fertilizers externally in their fields to maintain higher crop yields (Dimkpa et al. 2020). However, the excessive N application incurs higher environmental and farmer inputs costs, with sometimes human health implications (Ren et al. 2022). Moreover, crop plants

absorb only 30% of applied nitrogenous fertilizer (Pahalvi et al. 2021), and unutilized N is lost to water bodies and the atmosphere (Rahman et al. 2021). Presently, elevated demand and high cost of nitrogenous fertilizer in agriculture call for a "second green revolution," which could be accomplished by designing nitrogen-use efficient crops. These nitrogen-use efficient crops could efficiently uptake, translocate, and assimilate N and allow a lesser quantity of cascading reactive N to be released into the ecosystem (Liu et al. 2022b). Hence, this review has been formulated to focus on both present and forthcoming nitrogen use efficiency (NUE) for sustainable agriculture.

N is essential for plant growth and development (Liu et al. 2022a; Muhammad et al. 2022), and it is a constituent of essential biomolecules such as nucleic acid, enzymes, protein, ATP, chlorophyll, and alkaloids. Also, N improves root system architecture, resulting in better water and nutrient absorption from the soil. (Jia et al. 2022; Sinha et al. 2020a). In chronic N deficiency, older leaves undergo senescence and detach from plants (Zakari et al. 2020). Moreover, a deficiency of N leads to altered metabolism in the plant (Saloner and Bernstein 2021) and ultimately results in reduced yield and biomass (Gong et al. 2019; Meise et al. 2019).

The movement of N in the environment is governed by the biogeochemical cycle, and it involves the dynamic interaction of soil and plant systems. The interaction is regulated by various processes, including N fixation, nitrification, denitrification, and leaching. (Liu et al. 2022a). The flow of chemical N in the environment leads to environmental deterioration (Huang et al. 2019). The primary reason for N leaching in the ecosystem is the low NUE of crop plants (Darjee et al. 2023). The rise of chemical N in the soil causes groundwater pollution (Shukla and Saxena 2020. The enrichment of inland and marine water bodies with nutrients, especially N and phosphorus (P), in water bodies causes eutrophication, and a rise in greenhouse gases like N_2O contributes to global warming (Lenhart et al. 2019).

Genetic Approaches for Enhancing NUE

Various approaches are being utilized to enhance NUE to counteract the effects of low NUE on crop plants, economic yield, and the environment. The most prominent and efficient available method among the contemporary approaches includes conventional breeding and genetic manipulation of the transcription factors, transporters, assimilatory, and remobilization genes for nitrogen metabolism in crop plants to enhance the NUE (Lee et al. 2021; Kasemsap and Bloom 2022; Yadesa 2022).

The NUE is an inherently complex trait involving gene interaction networks that regulate N uptake, assimilation, translocation, and storage (Fig. 1). The NUE is governed by complex genetic makeup that mediates the N uptake and utilization (Mahboob et al. 2023). Several candidate genes in crop plants have been identified for improving NUE (McAllister et al. 2012; Li et al. 2022b), and through genetic manipulation, these identified genes have been modulated in crop plants to enhance NUE and crop productivity (Tegeder and Masclaux-Daubresse 2018; Luo et al. 2023; Table 1). In this regard, to the present date, many plant N–metabolism-associated transporters and assimilation enzymes have been overexpressed in crop plants resulting in enhanced plant growth even under N insufficient conditions (Fiaz et al. 2021; Yadav et al. 2021; Lal et al. 2023). Furthermore, it is critical to figure out the other probable limiting

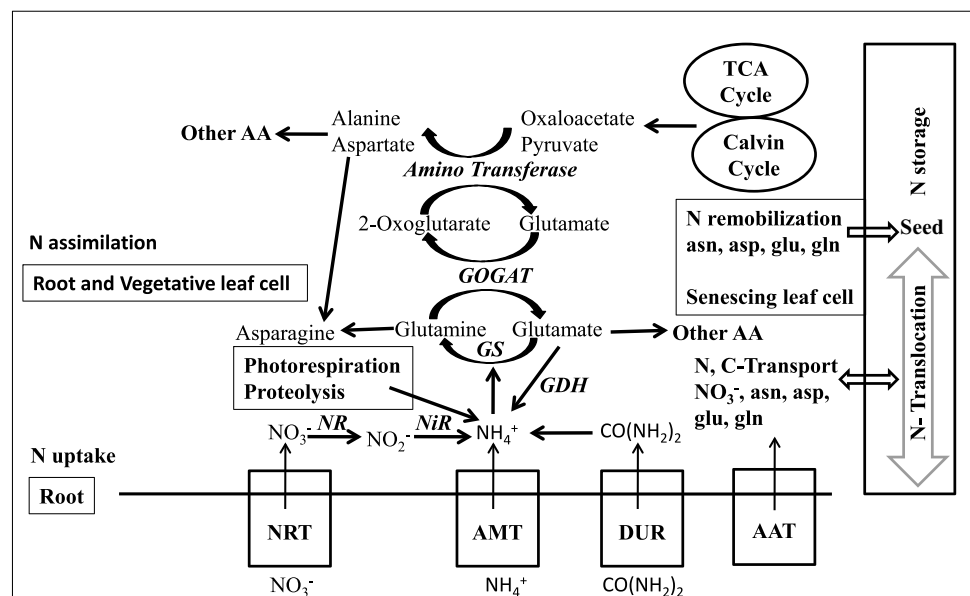**Fig. 1** Schematic pathway depicting nitrogen uptake, assimilation, and translocation

Table 1 Genes involved in NUE in crop plants

Gene	Gene product	Gene IDs	Function	Plant species	References
OsPTR6, OsPTR9	PTR/NRT1 transporter	Os04g0597800, Os06g0706400	Nitrate transport	Rice	Fan et al. (2014), Fang et al. (2013)
OsNRT2.3b, OsNPF7.3, OsNRT1.1A, OsNRT2.1, OsNRT1.1b	Nitrate transporter	Os01g50820, Os04g0597800, Os03g0235900, Os02g38230, Os03g0235900	Nitrate transport	Rice, barley	Luo et al. (2020), Wang et al. (2018), Fang et al. (2017), Fan et al. (2016a, b), Zhang et al. (2022)
OsMYB305	Transcriptional activator	Os01g0637800	Activate expression of a high-affinity nitrate transporter and nitrite reductase	Rice	Wang et al. (2020)
OsNRT2.3a, OsNAR2.1	Nitrate transporter and the accessory protein	Os02g0595900	Uptake and transport of NO_3^- and NH_4NO_3	Rice	Chen et al. (2020)
ZmAMT1;1a	Ammonium transporter	Zm00001eb426350	Transport of ammonium	Maize	Zhao et al. (2018)
HvALT	Alanine aminotransferase	LOC103835961, Os07g0108300	Carbon and Nitrogen metabolism	Mustard, rice, wheat, barley, sugarcane	Tiong et al. (2021), Shrawat et al. (2008), Good et al. (2007), Snyman et al. (2015)
OsAAT	Aspartate aminotransferase	Os12g0194900	Amino acid metabolism	Rice	Zhou et al. (2009)
OsTOND1	Tolerance of Nitrogen Deficiency1	Os12g0630100	Confers tolerance to the nitrogen deficiency	Rice	Zhang et al. (2015)
PsAAP1	Amino acid permease	LOC127084108	Amino acid transport	Narbon vetch and pea	Rolletschek et al. (2005), Grant et al. (2021)
ZmSPS	Sucrose phosphate synthase	AY468362.2	Sucrose biosynthesis	Alfalfa	Kaur et al. (2019)
HvGS1;1	Glutamine synthetase	AT5G57440	Ammonia assimilation	Barley	Gao et al. (2019)
OsPEPC	Phosphoenolpyruvate carboxylase	Os01g0208700	Carbon metabolism	Wheat, rice	Peng et al. (2018), Masumoto et al. (2010), Behera et al. (2023)
TaDof1	Transcription factor	AT1G51700	Nitrogen and carbon assimilation	Wheat	Hasnain et al. (2020)
OsGS1.1, OsGS2	Glutamine synthetase	LOCOs02g50240, LOCOs04g56400	Ammonium ion assimilation	Rice	Lal et al. (2023)
Osflo12	Floury endosperm12	Os10g0390500	Encode alanine aminotransferase	Rice	Zhong et al. (2019)
OsASN1, ZmTHP9	Asparagine synthetase	Os03g0291500, Zm00001d047736	ATP-dependent amidotransferase	Rice, maize	Lee et al. (2020b), Huang et al. (2022a, b)

Table 1 (continued)

Gene	Gene product	Gene IDs	Function	Plant species	References
OsNLP1, OsNLP4, AtNLP7	NIN-like protein	Os03g0131100 Os09g0549450	Orchestrates the expression of nitrogen uptake, assimilation, and signalling genes	Rice, cotton	Wu et al. (2021), Alfatih et al. (2020), Jan et al. (2022)
TaTAR2.1-3A	Cytokinin (CK) response regulator gene	TraesC-S3D02G246700	Auxin biosynthesis gene	Tobacco	Shao et al. (2017), Teng et al. (2022)
OsRuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase	Os02g0152400	The crucial role in carbon and nitrogen metabolism	Rice	Yoon et al. (2020)
OsMADS25	Transcriptional factors	Os04g23910	Lateral root development	Rice	Yu et al. (2015)

processes in N metabolism while engineering the crops for enhanced NUE in plants rather than N uptake under high N input agriculture. Advancements in genome editing tools like CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated nuclease 9) technology could be utilized for enhancing NUE in plants (Tiwari et al. 2020).

The key assimilatory genes include *glutamine synthetase* (*GS*), *Glutamine oxoglutarate aminotransferase* (*GOGAT*), *Alanine aminotransferase* (*AlaAT*), *Glutamate dehydrogenase* (*GDH*), *Aspartate aminotransferase* (*AspAT*), and *Asparagine synthase* (*ASN*) play a vital role in plant N metabolism (Lebedev et al. 2021; Singh et al. 2022; Fig. 1). Further, transcription factors (TFs) include *OsMAD25*, *OsDOF18*, *OsMYB305*, and N transporter like *NRT1*, *NRT2*, *AMT*, *AAP*, and *STP* are intensively characterized for improving NUE in crop plants.

Breeding Approaches for Enhancing NUE

Plant breeders aim to develop crop cultivars that can efficiently uptake N from the soil and its utilization inside the plant system. The NUE is the economic yield per unit N supplied to the plant. In contrast, physiological N use efficiency (PNUE) is the yield in relation to the amount of N accumulated within the plant as shown in Fig. 2 (Moll et al. 1982). PNUE is being applied in rice to assess NUE (Huang et al. 2022a, b). There is the existence of genetic variabilities in cereal crops for NUE traits as revealed by field studies (Pujarula et al. 2021). At the time of grain filling in plants, carbon (C) and N substrates are required, and N molecules get remobilized from shoots to the grain (Hajibarat and Saidi 2022). About 64% of remobilized N comes from the leaf blade and leaf sheath, while the stem contributes 36% of remobilized N from vegetative tissue to panicle at the time of grain filling (Mae and Ohira 1981). Significant variation exists among cultivars for N remobilization during the reproductive stage, and prevailing variation in rice germplasm could be employed for efficient N management (He et al. 2021). NUE is well correlated with the translocation of metabolites from leaves to panicles during grain development in rice (Melino et al. 2022).

During the Green revolution era (1960s), semi-dwarf wheat and rice varieties were bred to exploit the higher doses of nitrogenous fertilizers resulting in higher yield and productivity. However, in the last few decades, excessive use of nitrogenous fertilizer has witnessed yield stagnation along with environmental hazards, which brought much attention to the plant breeders for developing N-use efficient crop varieties (Liu et al. 2022b).

NUE is defined broadly as a dual function of N uptake efficiency (NUpE) and utilization efficiency (NUtE). The
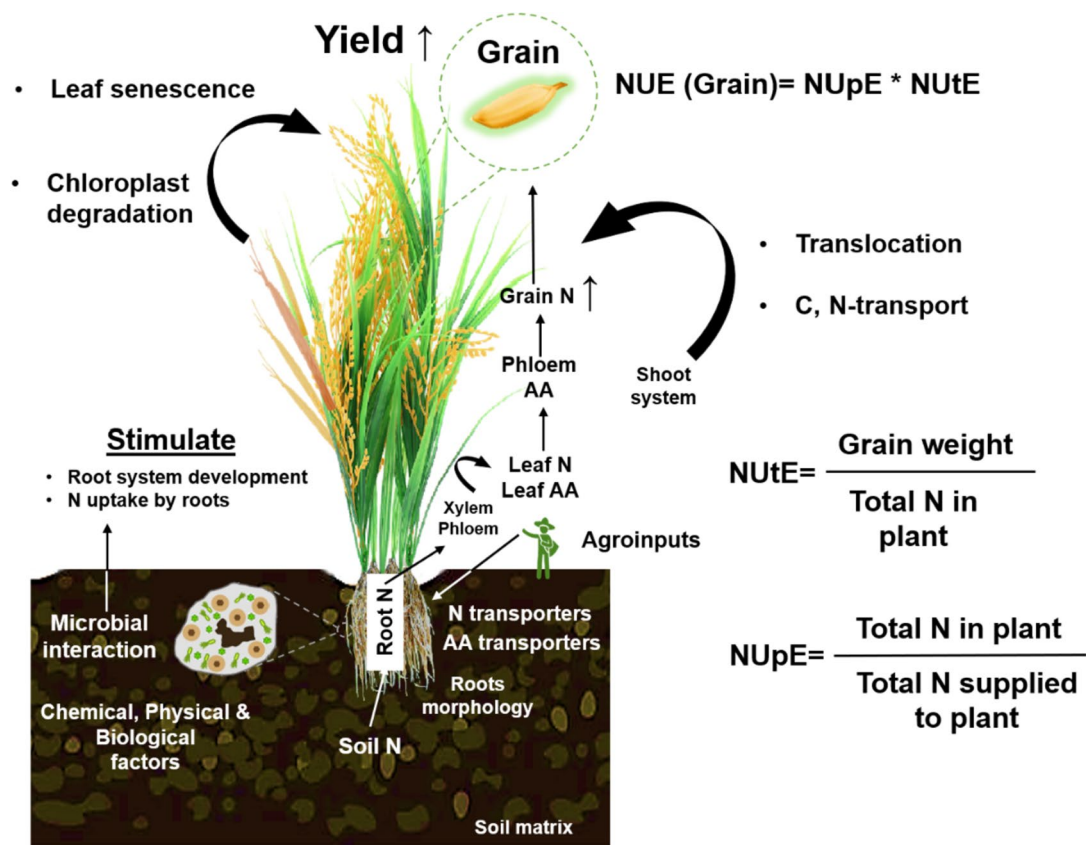
Fig. 2 Different processes involved in nitrogen uptake, utilization, and NUE

availability of adequate genetic variability for component traits is a prerequisition for response to genetic gain under selection. Classical plant breeding methods have been wonderfully performed in the last three decades by selecting the most desirable features in terms of yield improvement. However, very little attention has been paid to understanding the physiological and genetic basis, particularly in relation to NUE. Most of the plant breeding efforts have been made under idealized conditions to enhance productivity, and it is quite obvious that valuable allelic diversity might have been significantly reduced in available breeding materials for tolerance to limiting N conditions.

In the past, most peer-reviewed research papers for important crops suggested that contemporary cultivars have sufficient genetic variation for selecting and enhancing NUE component traits (Hawkesford 2017; Hawkesford and Griffiths 2019). Another source of genetic diversity can be found in the secondary or tertiary gene pool or wild relatives of almost all other major food crops, and NUE has not been thoroughly characterized in these potential wild cultivars (Hawkesford 2017). therefore, there is a greater scope for the identification and introgression of the NUE-related genes in elite cultivars through backcross breeding programs.

Grain yield and biomass are primarily used to select promising phenotypes under low N and optimal N conditions. Despite the availability of adequate variability for the component traits under low N, lower overlapping values in normalized conditions make the selection more attractive (Gallais et al. 2006). However, direct selection under low input/low N status yielded desired results, mainly in wheat and maize (Presterl et al. 2003; Laperche et al. 2006). Simultaneous selection of genotypes based on grain yield in both N stress and optimal conditions has resulted in the overall improvement in both conditions. Designing an efficient breeding program is based on the kind of genetic materials, genetic parameters of variability, and stability of parameters across the environment. The ability of the genotype to efficiently take up N is a key component trait for plant NUE. The key traits for NUE include N uptake at stress conditions, harvest index (HI), N harvest index (NHI), and biomass potential are important for a breeder while developing nitrogen-use efficient lines.

Genetic variability for root size, distribution, and proliferation has been reported in several crop plants; however, it also varies with soil types and nutrient status (Matsunami et al. 2009, 2010). Genetic diversity for root traits was observed while screening spring wheat genotypes

under pot and hydroponics conditions and they have found NU_pE largely responsible for contributing towards NUE mainly because of high root proliferation and biomass production (Ranjan et al. 2019). Sufficient variability for NUE in modern wheat cultivars was observed under low N conditions (Hawkesford 2017). However, very little variation for grain yield suggests incorporating a more diverse range of genotypes for creating a wider range of variability for yield coupled with NUE. Some previous research with N metabolism and signalling pathway-governed genes show adequate variability in crop species for breeding the NUE efficient cultivars. The most striking variability of NUE-related genes in rice is for a GOGAT enzyme. This gene was originally reported in *japonica* rice and transferred in *indica* background and resulting in enhancement of yield (Yamaya et al. 2002).

Since NUE is an integrative complex trait, decided by the number of key traits and other morpho-physio traits that are linked or function independently. The presence of significant genotypes by environment (G × E) interactions reduces the overall effectiveness of selection using grain yield under low N conditions. In addition, the polygenic nature, linkage, and non-additive gene action coupled with low heritability further complicate the selection criterion. Indirect selection using the component traits is more realistic because of high heritability and more stability with respect to genetic and external environmental factors. The selection-based index is the most attractive approach for complex traits like NUE; it overcomes the limitation of single trait selection and allows simultaneous improvement of component traits. The appropriate selection of selection index may vary depending upon the conditions and usefulness of the breeders while working on a particular problem. N efficient wheat lines identified based on utilizing four selection indices: tolerance index (TOL), stress susceptibility index (SSI), geometric mean productivity (GMP) and yield susceptibility index (YSI) (Tyagi et al. 2020). Presently, conventional breeding methods have been greatly improved by integrating the knowledge of genetics, physiology, and biochemistry.

Exploiting genetic variability through innovative molecular breeding technology is in great demand because of the easy dissection of complex traits and assisting in phenotyping in less time. Precise phenotyping of NUE traits is cumbersome and requires vigorous exercise to monitor the component's traits. Advancement in developing next-generation markers/sequencing-based markers and bioinformatics improves the chances of locating and mapping QTLs governing the NUE traits. While breeding nitrogen-use efficient cultivars, key agronomic traits such as grain protein content, grain yield, test weight, and other NUE traits such as NHI, N remobilization capacity, and N content in grain were used in the breeding program (Karunarathne et al. 2020).

Molecular linkage genetic maps and quantitative trait locus (QTL) mapping technologies are commonly used to estimate loci's number and precise location governing genetic variation in various genetic materials (segregating or fixed populations). The selection of appropriate parents, evaluation of effective population sizes, multi-location testing, and the availability of high-density genetic maps are all required for successful QTL mapping programs for complex traits like NUE (Han et al. 2015). N deficiency traits (NDT) have also been targeted as an indirect selection criterion for selecting nitrogen-use efficient phenes in rice (Shen et al. 2021), and several QTLs for NDT and NUE traits in rice have been identified. (Ogawa et al. 2016; Shen et al. 2021). A few of a total of six QTL contributing towards NUE or low N-responsive traits in winter wheat showed the association with photoperiod and disease resistance and suggested for exploitation of potential marker-assisted selection (MAS) for breeding soft red winter wheat (Brasier et al. 2020).

Li et al. (2015) identified 184 and 147 QTLs for NUE and root system architecture (RSA) related traits and established a genetic relationship with RSA. They also explored the genomics segment for MAS in maize to improve complex traits like NUE and others. A total of 15 QTLs showing significant effects under low N conditions were identified in a barley population (Prisma × Apex) (Kindu et al. 2014). Besides that, several other QTLs have also been identified through genome-wide association studies (GWAS) for key indicator traits of NUE in barley (Pasam et al. 2012). Fifty-two putative QTLs were reported for different NUE traits in a double haploid (DH) potato population and field trial at several locations with different N-levels, specific QTLs showing significant QTL × E interaction (Getahun et al. 2020).

NUE and its component traits are mostly polygenic and background-specific; thus, the ultimate target would be to converge all the small effect loci in one genetic background to advance the realized genetic gain (R). Genomic selection is one of the most appropriate strategies to target all the small-effect minor QTLs through a predictive equation based on genome-estimated breeding value (EBV) and facilitate overall genetic improvement of complex traits like NUE. In future research, there is a need to holistically target the key information on the agronomical aspect of NUE, physiological NUE, and absorption NUE in respect of breeding varieties for low input or low N conditions.

Engineering Root System Architecture for NUE

The gene reported to control root growth and to give a particular response towards nutrients in soil has shown to be a promising target for engineering plants for better nutrient use

efficiency. *OsTOND1* belongs to QTL, which confers tolerance under N deficiency in rice. It codes for a thaumatin protein and its role in the induction of primary root elongation in low N soil. *OsTOND1* has been found in only some *indica* rice varieties, which is absent in the *japonica* rice varieties studied so far. Furthermore, overexpression of *OsTOND*1 in rice confers tolerance to nitrogen deprivation, and overexpressed rice plants growing in nitrogen-deficient conditions have increased shoot N content, grain production, panicle number, dry weight, chlorophyll content, plant height, and root length (Zhang et al. 2015; Sujata et al. 2019).

A longer root system meant for the acquisition of nutrients from deeper soil layer, a characterized QTL, DEEPER ROOTING1 (*DRO1*) responsible for longer root in upland land rice, affects root system architecture in *Arabidopsis* and *Prunus* species and also enhances yield under drought in rice (Uga et al. 2011, 2013; Arai-Sanoh et al. 2014; Guseman et al. 2017). The rice cultivar with the DRO1 gene exhibiting enhanced N uptake and grain yield increased by 10% compared to the rice variety with a dormant *DRO1* allele (Arai-Sanoh et al. 2014; Kitomi et al. 2020). Rice transcription factor *OsMADS25* is involved in N signalling and it is predominantly expressed in the root during the growth period. *OsMADS25* overexpression in rice results in better root system architecture and higher shoot mass (Yu et al. 2015; Nazish et al. 2021).

Mild N deficiency enhances lateral root (LR) branching (Forde 2014). A study in *Arabidopsis* reveals that N deficiency causes an increase in lateral roots (Ma et al. 2014). Moreover, expression of tryptophan aminotransferase related 2 (*TATAR2*) was enhanced under low nitrogen in wheat, and knockdown mutant of *TATAR2.1* exhibits impaired lateral root growth and phenotype defects under limiting and optimum N supply in wheat (Shao et al. 2017). Also, *TaTAR2.1-3A* overexpression in wheat enhanced plant growth, grain yield, primary root length, lateral root branching, and N accumulation at different N conditions while reducing expression of *TaTAR2.1* have opposite effects (Shao et al. 2017; Teng et al. 2022).

Genetic Manipulation of N Transporters

The N transporters play a crucial role in N plant metabolism. Moreover, N transporters are extensively studied in rice, and its genome encodes 94 nitrate transporter 1 (*NRT1*)/peptide transporter (PTR), five nitrate transporter 2 (NRT2), five chloride channel (CLC), and nine slow anion channel-associated homologs (*SLAC/SLAH*) gene (Lee 2021). Transport of NO_3^- inside crops is mainly regulated through *NRT1* and *NRT2* transporters (Aluko et al. 2023). However, utilizing existing natural variations and overexpressing NO_3^- transporters resulted in enhanced NUE and grain yield in rice

(Hu et al. 2015; Fan et al. 2016b; Zhang et al. 2022). The *OsNPF7.6* is a member of the nitrate transporter 1/peptide transporter family (*NPF*) expressed in all plant tissues. Moreover, overexpression of *OsNPF7.6* enhances the rice yield and NUE (Zhang et al. 2022). Furthermore, *OsNPF7.3* of the NO_3^- *NPF* is expressed in lateral roots and stem and localized in the vacuolar membrane. The elevated expression of *OsNPF7.3* in rice results in enhanced grain yield, tiller number, and grain N content (Fang et al. 2017). The NH_4^+ induces the expression of *OsNRT1.1A*, which belongs to the NO_3^- transporter1 family. In rice, *OsNRT1.1A* overexpression results in up to 50% enhancement in NUE, grain yield, and early maturity by 18 days. On the contrary, the *OsNRT1.1A* mutant shows growth retardation, yield loss, late flowering, and reduced utilization of NH_4^+ and NO_3^- (Wang et al. 2018).

Studies revealed diversification in rice genotypes for uptake efficiency of NO_3^-; the *indica* subspecies have higher NO_3^- uptake efficiency than the *japonica* subspecies that is due to variation of the gene sequence of *OsNRT1.1B* (Hu et al. 2015). Moreover, *OsNRT1.1b* overexpression in rice accumulated higher N and improved plant growth under low and sufficient N supply (Fan et al. 2016a). Moreover, rice contains a high-affinity transporter, *OsNRT2.3* consisting of *OsNRT2.3a* and *OsNRT2.3b,* splice forms that code for plasma membrane protein. *OsNRT2.3a* function in association with nitrate transport accessory protein, *OsNAR2.1* (Yan et al. 2011; Tang et al. 2012). The concurrently expressing *OsNAR2.1* and *OsNRT2.3a* in rice results in enhanced uptake and transport of NO_3^- and NH_4^+, and the grain yield and NUE were enhanced by 24.6% and 28.6% in co-overexpressed lines (Chen et al. 2020). Also, overexpression of *OsNRT2.3b* in barley results in up to 43% enhancement in NUE. The grain weight and yield of overexpressed lines are higher than control plants (Luo et al. 2020). Furthermore, overexpression of *OsNRT2.3b* in rice results in up to 40% higher NUE and grain yield (Fan et al. 2016b). The study reveals that *NRT2* plays a key function in increasing yield and NUE under a different N supply. However, the gene should be expressed with tissue-specific promoters to get the desired result.

The *OsPTR6* and *OsPTR69* belong to the *PTR/NRT1* family. The *OsPTR6* overexpression in rice enhances the activity of glutamine synthetase (GS), NUE, and growth of the plant as compared to control plants supplied with NH_4^+ at the optimum amount (Fan et al. 2014), and overexpression of *OsPTR9* in rice enhances NH_4^+ absorption, which drives LR branching and boosts grain production. On the contrary, *OsPTR9* T-DNA mutant and *OsPTR9* RNAi lines exhibit growth retardation (Fang et al. 2013).

Ammonium is transported inside plants through ammonium transporter (AMT). Rice (*Oryza sativa*) genome encodes four AMT family members: *OsAMT1*, *OsAMT2*,

OsAMT3;1, *OsAMT3*, and *OsAMT4*. The *OsAMT1* members belong to high-affinity transporters, while the other three family members are low-affinity transporters (Wu et al. 2017). Crop plants primarily absorb N in NH_4^+ and NO_3^- forms. In rice, overexpression of *OsAMT1-1* boosts NH_4^+ uptake and its translocation inside the plant at an optimum level, but under a high amount of NH_4^+ supply, it impairs plant growth (Ranathunge et al. 2014; Li et al. 2016). Also, overexpressing *OsAMT1.3* in rice enhances plant growth, and plants able to adapt at a low concentration of NH_4^+ (Bao et al. 2015; Ferreira et al. 2015). In rice, activation tagging mutants co-expressing *OsAMT1;2* and *OsGOGAT* had better grain yield and NUE under deficient N as that of wild type and double mutants of *OsAMT1;2* and *OsGOGAT1* exhibit higher protein and N concentration as compared to wild type (Lee et al. 2020a). Also, *ZmAMT1;1a* overexpression in maize shows a 17% increase in NH_4^+ uptake under low NH_4^+ supplied to plants as that of control plants (Zhao et al. 2018).

N uptake in crop plants is regulated by transcription factors (TFs) and signalling molecules (Zhang et al. 2020; Zuluaga and Sonnante 2019). Among the TFS, DNA binding with one finger 18 *(OsDOF18)* rice mutant has impaired NH_4^+ assimilation and reduced expression of $NH4^+$ transporter genes. Rice mutant plants grow well on supplying NH_4^+ as NO_3^- (Wu et al. 2017) and N-mediated heading date-1 (*Nhd1*) activate the transcription of *OsAMT1;3* and *OsNRT2.4* and knockdown of the *Nhd1* inhibits the root growth in rice (Li et al. 2022a).

The *OsMYB305* encodes for transcription activator in rice. Under low nitrogen, this gene shows higher expression in the root. The overexpression of *OsMYB305* in rice exhibits enhancement in tiller number, shoot dry weight, and increased N accumulation in the plant grown under low N conditions. The expression of a high-affinity NO_3^- transporter and nitrite reductase gene upregulated in *OsMYB305* overexpressed lines (Wang et al. 2020).

The NIN-like protein (NLP) is involved in nitrate signalling by regulating the expression of several N uptakes and assimilatory genes (Mu and Luo 2019). The *OsNLP4* overexpression in rice results in an enhancement in yield by 30% and increased NUE by 47% as that of the control plant. The loss of function rice mutant generated through CRISPR/Cas9 shows a significant reduction in yield under low nitrogen (LN), normal nitrogen (NN), and high nitrogen (HN) as that of wild type (Wu et al. 2021). Also, overexpressing *OsNLP1* in rice improved the biomass, grain yield, and NUE on supplying with variant N supply. The knocking out of *OsNLP1* reduces the grain yield and NUE (Alfatih et al. 2020). Moreover, Overexpression of *AtNLP7* upregulates the expression of N uptake and assimilatory genes and improves the NUE and yield in cotton (Jan et al. 2022).

Genetic Manipulation of Key Genes of C and N Metabolism

The well-known *RuBisCO* plays an important role in C fixation by incorporating CO_2 into Ribulose 1,5-bisphosphate (RuBP) and converting it into 3-phosphoglycerate (3-PGA). At a low concentration of CO_2, RuBP combines with O_2 to produce phosphoglycolate and later releases ammonium ions during photorespiration, hence affecting carbon fixation (Stitt et al. 2010). In C3 plants, released ammonium ions are returned to amino acid pools through assimilatory enzymes (McAllister et al. 2012). At a higher level of CO_2, plants exhibit enhanced assimilation of N and C (Zong and Shangguan 2016). Knocking down the *RuBisCO* expression in crop plants affects the C and N metabolism, reducing grain yield, biomass, amino acid pools, and plant metabolites (Maheshwari et al. 2021).

Furthermore, overexpression of *RuBisCO* in rice causes an increase in photosynthesis and NUE under sufficient N fertilization (Yoon et al. 2020). *PEPC* (phosphoenolpyruvate carboxylase) is another enzyme involved in photosynthesis and N storage in plants; this enzyme is involved in vital metabolic processes in bacteria, algae, and other lower species. The product of this enzyme is oxaloacetic acid (OAA), having a vital role in other metabolic processes like tricarboxylic acid cycle (TCA) cycle, amino acid biosynthesis, gluconeogenesis, urea cycle, glyoxylate cycle, fatty acid synthesis, etc. (Doubnerová and Ryšlavá 2011). *PEPC* plays a crucial role in N assimilation as validated by the knockdown of chloroplastic *PEPC* isoforms (Masumoto et al. 2010). In rice, *PEPC* overexpression enhances photosynthesis, biomass, and grain yield (Behera et al. 2023). Moreover, *ZmPEPC* overexpression in wheat results in upregulating TCA cycle genes that signify more C generation under low and high N conditions. The total amino acid concentration was enhanced by 48.18% in overexpressed lines as that of wild-type plants (Peng et al. 2018).

Plants mostly uptake inorganic N in the form of NO_3^- and NH_4^+ from the soil, while plants take up organic N in the form of amino acids through amino acid transporter (AAT). The transporters are classified into three major families: ATF (amino acid transporter family), APC (amino acid-polyamine-choline transporter family), and UMAMIT (usually multiple acids move in and out transporter family). Among all these families, most research has focused on AAP (amino acid permeases) because of their multiple physiological functions, AAP transporters belong to ATF (Yang et al. 2020). The *AAP1* gene overexpression is extensively studied in legumes and *VfAAP1,* specifically overexpressed in the seed of *Vicia narbonensis* and *Pisum sativum* results in up to 15% increase in

total N content and enhancement in seed size up to 30% and also, co-overexpression of *AAP1* and sucrose transporter, *SUT1* in pea results in enhanced grain number, protein and sugar content (Grant et al. 2021; Rolletschek et al. 2005). Sugar transporters are associated with phloem loading and unloading in plants, and these transporters are involved in carbon distribution inside plants (Wen et al. 2022). Sugar transporter proteins (STPs) play crucial roles in plant sugar transport. Overexpressing *STP13* (hexose transporter) in *Arabidopsis* results in an enhancement in glucose uptake, internal glucose amount, and biomass when plants are grown in a limiting N environment (Schofield et al. 2009). These studies suggested a link exists in plant systems for coordinating C and N metabolism. Improvement in NUE requires both C substrate and N availability, the lack of C substrate can compromise the NUE, and N availability affects C fixation (Sandhu et al. 2021). The GS/GOGAT cycle is also critical for maintaining the C/N ratio. The keto group contains amino acids involved in C metabolism utilizing glutamate as signalling molecules (Hildebrandt et al. 2015). The tuning of the C/N ratio is crucial for plant growth and development. Moreover, the NUE is regulated by the photosynthesis assimilation rate, and under the limitation of C substrate, NUE gets reduced (Liu et al. 2022a).

The primary enzyme in sucrose production is sucrose phosphate synthase (*SPS*). The overexpression of *SPS* in alfalfa plants results in an enhancement in growth and nodule development, and overexpression of *SPS* in sugarcane enhances growth and increases the height, sucrose and biomass content (Kaur et al. 2019; Anur et al. 2020). N assimilatory gene, *AlaAT* converts pyruvate to alanine, and this conversion is activated during hypoxia; thus, *AlaAT* links Glycolysis and TCA Cycle (Rocha et al. 2010). Further, the genetic manipulation of *HvAlaAT* using root and stress inducible promoter enhances NUE and grain yield in rice, barley, wheat, oilseed rape (*Brassica napus*) and sugarcane under low N conditions (Tiong et al. 2021; Good et al. 2007; Shrawat et al. 2008; Snyman et al. 2015).

The *Dof* gene of maize has been utilized for improving N assimilation by regulating the TCA cycle. The overexpression of *Dof1* in rice enhances the C flow, which results in improved plant growth under low nitrogen (LN) environments (Kurai et al. 2011). Also, overexpression *TaDof1* in wheat shows enhanced expression of citrate synthase, phosphoenolpyruvate carboxylase, isocitrate dehydrogenase, pyruvate kinase, and overexpressed wheat transgenic lines show improved agronomical traits under limiting N supply as compared to wild type (Hasnain et al. 2020).

Amino Acid Metabolism Regulates NUE

Glutamine synthetase (*GS*) catalyzes the ATP-dependent condensation of ammonia and Glutamate (Glu) to Glutamine (Gln), and GOGAT transfers the amide group of Gln to 2-oxoglutarate to form two molecules of Glu. Plant encodes two forms of GS, the cytosolic (*GS1*) and chloroplastic (*GS2*). In plants, GOGAT exists in two ferredoxin-GOGAT (*Fd-GOGAT*) and *NADH-GOGAT* that are expressed in chloroplast and cytoplasm, respectively. GS enzyme is the prime target of researchers for enhancing the NUE of crop plants (Hakvoort et al. 2017). GS modulated in plants through genetic engineering and tobacco plants overexpressing *Arabidopsis GS1*, *GS2* grown under low nitrogen exhibits higher sugar, protein, and chlorophyll content (Wang et al. 2013). The cisgenic *HvGS1;1* overexpressed line of barley exhibited enhanced grain yield and NUE compared to control plants at different N-supplied (Gao et al. 2019). Studies in durum wheat reveal GS2 alleles co-localized with grain protein content QTL, which makes the possibility of GS2 imparting a role in enhancing grain protein content (Gadaleta et al. 2011). Also, GS-overexpressed rice contains enhanced protein, amino acid, and total nitrogen content compared to wild type (Cai et al. 2009). Moreover, transgenic wheat expressing the plastidic *GS2* isoform under low and high N regime conditions shows higher yield, NUE, and grain N concentration like wild type (Hu et al. 2018). Furthermore, Concurrent co-overexpression of *OsGS1;1* and *OsGS2* increased NUE, yield, and biomass in *indica* rice (Lal et al. 2023).

Plant roots exhibit plasticity rather than preference in the acquisition of NH_4^+ and NO_3^- (Chalk and Smith 2021). On supply of NH_4^+ to *Arabidopsis*, *NADH-GOGAT* shows higher expression and accumulation in the roots. T-DNA mutant line having non-functional *NADH-GOGAT* shows a reduction in biomass when the major N source is supplied as NH_4^+ (Konishi et al. 2014). In addition, glutamate dehydrogenase (*GDH*) is also a key enzyme in ammonia assimilation that converts 2-oxoglutarate to glutamate. The rice genome encodes four putative genes for Glutamate dehydrogenase (*OsGDH1-4*). There is differential regulation of *OsGDH* family members that takes place under P and N deprivation (Qiu et al. 2009). Overexpression of Novel *TrGDH* (*Trichurus GDH)* in rice results in improved plant growth and grain weight per plant (Du et al. 2019).

Aspartate aminotransferase (AAT; aspartate:2-oxoglutarate aminotransferase) catalyzes the reversible transamination reaction of glutamate and oxaloacetate to form aspartate and 2-oxoglutarate. AAT plays a key role in the C and N metabolism in plants. In rice, overexpression of aspartate aminotransferase *(AAT)* using CaMV 35S promoter enhanced

AAT activity in the leaf and higher protein and seed amino acid content (Zhou et al. 2009). Another enzyme, asparagine synthetase (ASN) forms asparagine by condensing ammonia and aspartate or through the transamination of glutamine and aspartate. In *Arabidopsis*, ASN is encoded by three genes: *ASN1*, *ASN2*, and *ASN3* (Gaufichon et al. 2016). In *Arabidopsis*, Asparagine synthetase 2 (*ASN2*) overexpression leads to amino acid accumulation under NH_4^+ as a sole N source, which depicts the role of *ASN2* in NH_4^+ detoxification and N assimilation. Asparagine content was reduced in plants that under-express *ASN2* (Igarashi et al. 2009). On overexpressing *OsASN1* in rice enhanced the expressions of *OsAMT1;1*, *OsAMT1;2*, and *OsAMT1;3*. In overexpressed lines, Asparagine content in grain was enhanced to 3.4-fold, and N content in grain increased to 140% as that of wild type. (Lee et al. 2020a, b). Also, asparagine synthetase 4 (*ZmTHP9*) overexpressed maize lines exhibit enhanced asparagine content and seed protein content (Huang et al. 2022a, b). Furthermore, alanine:2-oxoglutarate aminotransferase (AlaAT) catalyzes the reversible transamination of glutamate and pyruvate to produce 2-oxoglutarate and alanine in the plant cytoplasm and mitochondria. Map-based cloning reveals floury endosperm12 (*flo12*) encodes alanine aminotransferase1 (*OsAlaAT1*) in rice. However, the starch synthesizing genes downregulated in *flo12* mutants. Further, the amylose content was reduced, and protein content was enhanced in *flo12* grain. This study depicts how *OsAlaAT1* regulates the C and N metabolism (Zhong et al. 2019).

Future Prospects

For developing nitrogen use-efficient crops, the genes involved in N uptake, mobilization, and translocation need to be stacked and expressed inside the crop plants (Fig. 3). For enhancing NUE in crop plants, tissue-specific, inducible promoters could be utilized. Further, studies have revealed that enhancement in NUE is linked with C metabolism (Behera et al. 2023; Jin et al. 2022; Rosolem et al. 2022). Therefore, the C and N metabolism genes could be stacked together to get desired and consistent results.

The microbial communities are involved in biological N fixation and its uptake. For example, mycorrhiza mobilizes N and P and is also a reservoir of C (Scartazza et al. 2023). Additionally, the microbial genes involved in N fixation and uptake could be extensively screened, and desired NUE genes could be isolated from microbes. Furthermore, these genes can be transferred to crop plants for enhancing NUE. In addition to that, the mutant alleles imparting high NUE in crop plants could be made using CRISPR/Cas9 gene editing technology and mutant alleles imparting high NUE could be transferred in elite crop cultivars through conventional breeding. However, field trials of reported candidate genes associated with NUE need to be extensively conducted to reach at a final conclusion. Moreover, this study aids in understanding recent advancements and developments in improving crop NUE and yield. Therefore, the genetic approaches mentioned in
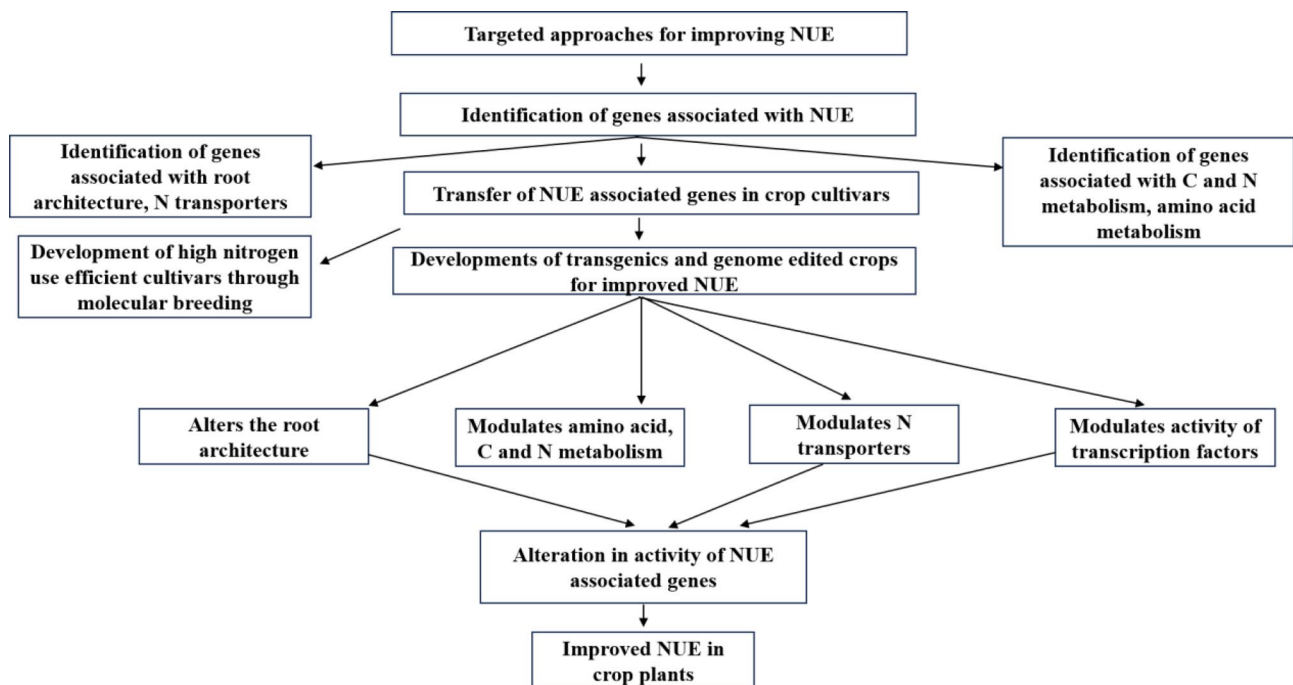

Fig. 3 Targeted approaches for improving NUE in crop plants

this study can be adopted to obtain "tailored crop plants" having efficient NUE capabilities.

Concluding Remarks

In the present scenario of high N input agriculture, one of the major bottlenecks that dampen the farmer's confidence and increase the agriculture–associated costs are the low NUE of crop plants (Govindasamy et al. 2023). Thus, in this scenario, one of the suitable solutions for maintaining crop productivity without increasing fertilizer usage is the development of crop plants with improved NUE via transgenic and gene editing technology (Karunarathne et al. 2022; Zhang et al. 2021; Sinha et al. 2020b; Fiaz et al. 2021). Moreover, intensive cultivation, adoption of high-yielding crop varieties, and hybrids in agriculture require enormous application of nitrogenous fertilizers in soils (Shukla et al. 2022). The N-efficient and responsive crop varieties need to be developed to reduce the nitrogen requirement. Developing such varieties is a bit challenging and complex task for the breeders. The identification and incorporation of potent genes, alleles, and QTLs for efficient nitrogen use efficiency into cultivated varieties through genetic engineering methods could be a promising alternative to minimize the use of fertilizers in agriculture (Karunarathne et al. 2022; Dong et al. 2022).

The cross-talk pathway related to nitrogen use efficiency needs to be modified to enhance NUE in crop plants. The carbon–nitrogen and nitrogen-phosphorus could be the prime targets for improving nitrogen use efficiency in crop plants. The most potent target for enhancing NUE could be the nitrogen transport pathways; the key transporters and activators of such pathways must be modified to channel nitrogen molecules into plant systems. Apart from this, the prime target could be the signalling pathway that directly/indirectly regulates nitrogen uptake, transport, assimilation, and translocation The geneticist and plant biotechnologist's role is crucial for modifying the pathway in a specific and tailored way. The key genes involved in the pathway could be stacked to get a better phenotype for nitrogen use efficiency in crop plants. The developed designer N uses efficient crop plants, so-called super crops could provide a path and road map for an evergreen revolution.

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Declarations

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